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FREEZE-FRACTURE STUDY OF WATER-SOLUBLE, STANDARD PROTEINS AND OF DETERGENT-SOLUBILIZED FORMS OF SARCOPLASMIC RETICULUM Ca^{2+}-ATPase

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Summary

Conventional freeze-fracture electron microscopy was used to study water-soluble proteins and different forms of Ca^{2+}-ATPase-detergent complexes. Freeze-fracture images of solutions containing proteins larger than myoglobin showed the presence of distinct, randomly dispersed particles on smooth fracture surfaces. The distribution of sizes of these particles was close to Gaussian, with a mean size which was correlated to the Stokes diameter. Monomeric Ca^{2+}-ATPase from sarcoplasmic reticulum, solubilized by deoxycholate or a non-ionic detergent, showed a bimodal distribution of particle sizes. Even more complex distributions were found for dimeric and trimeric preparations of Ca^{2+}-ATPase. The results can be interpreted on the assumption that the Ca^{2+}-ATPase molecule is elongated, with an overall length of about 110 Å and a width in its largest part of about 75 Å. It is concluded on the basis of the presented results that freeze-fracture electron microscopy can be successfully used for morphological studies of protein molecules in solution.

Introduction

Freeze-etching electron microscopy currently is used almost exclusively in the study of biological membranes or membrane model systems, such as liposomes or associations between lipids and proteins. There are few studies on

Abbreviations: SDS, sodium dodecyl sulphate; $C_{12}E_8$, dodecyl octaethyleneglycol monoether; Tes, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulphonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

other materials and, in particular, on biological macromolecules in solution. The main reason for this situation is probably the technical complexity of freeze-etching as compared to negative staining techniques, which in many cases are more appropriate for such studies. Negative staining cannot under ordinary circumstances be used in the study of protein-detergent complexes because of the high sensitivity of the complexes to the changes in ionic strength occurring during the staining procedure. Freeze-etching electron microscopy would be much more appropriate in this case, provided that the freezing can be performed rapidly enough to avoid any artefactual aggregation due to segregation of solute and solvent during the freezing period.

We have recently shown that freeze-etching electron microscopy can be successfully used to study the morphology of molecules in solution when ultrarapid cryofixation, well-controlled etching and improved shadowing are used [1,2]. In this paper we describe a much simpler method for studying water-soluble protein and detergent-protein complexes. The main purpose of this work is to show that conventional freeze-fracture electron microscopy can be successfully used to characterize water-soluble and detergent-solubilized proteins, and thereby provide information about their approximate dimensions and their monomeric or multimeric states in solution.

Materials and Methods

Standard proteins. In a preceding study we found that few commercially available proteins were suitable for calibration of gel columns when examined by a number of criteria: Elution as a single and narrow peak by gel chromatography, sedimentation as a single and homogenous boundary by analytical ultracentrifugation, and production of single peptide bands by SDS-gel electrophoresis. Most of the protein preparations which were found to be suitable were also used in this study (cf. Table I of Ref. 3): cytochrome *c* (Sigma C 7752), ribonuclease (Sigma R 5000), ovalbumin (Sigma A 2512), alkaline phosphatase (Sigma P 4252), transferrin (Sigma T 2252), aldolase (Boehringer), catalase (Pharmacia), β-galactosidase (Sigma G 6008). We also used *Escherichia coli* aspartate transcarbamylase, which we prepared according to the method of Gerhart and Holoubek [4]. The values used for the Stokes radii (R_s) of all the above-mentioned proteins were taken from Table I in Ref. 3. In addition we have used hemoglobin (Sigma H 7379), which at the high protein concentration used was found to be in an unambiguously tetrameric state ($s_{20,w}$ = 4.3 S, R_s = 32 Å) *. The proteins ordinarily were dissolved at a concentration around 10 mg/ml in either 30 mM Tris-HCl buffer (pH 8.3)/1 mM EDTA/1 mM NaN_3 or in 10 mM Tes (pH 7.5)/0.1 M KCl/0.1 mM $CaCl_2$.

Ca^{2+}-ATPase. Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle and extracted twice with a low concentration of deoxycholate to remove proteins other than the Ca^{2+}-ATPase from the membrane as described by Meissner et al. [5]. The preparation and purification of the soluble forms of the ATPase: monomers, dimers, trimers or tetramers was per-

* We previously found that the protein was in an essentially dimeric state when it was eluted at a low concentration from a gel column [3].

formed by gel chromatography as previously described in the presence of deoxycholate [6—8] or $C_{12}E_8$ [9]. (The results of a typical experiment, using deoxycholate, are presented in Fig. 5.) After elution from the gel column the ATPase samples were concentrated by Amicon Dia-flow filtration through an XM-50 filter. Fractions with the same size of ATPase were pooled and dithiothreitol was added to a concentration of 1 mM before concentration. In some cases the material was concentrated by centrifugation for 19 h at 120 000 × *g*, or by rotary evaporation in the case of $C_{12}E_8$-ATPase trimers. By these procedures the protein concentration was elevated approx. 10-fold to around 2—4 mg/ml as measured by the Biuret reaction [10]. In some cases we checked, by sedimentation velocity studies in the analytical ultracentrifuge, that the sample after concentration remained in the same state of aggregation as after elution from the gel column.

Freeze-fracture electron microscopy. The solutions and suspensions were prepared in buffers containing 25—30% of glycerol. Small drops of preparations were deposited on conventional Balzers gold planchettes and then frozen in liquid Freon-22 (at −160°C) or in liquid propane (at −196°C). Fracturing and replication were done using Balzer's BAF 301 apparatus in the conventional way. The replicas were cleaned in chromic acid, washed with distilled water and observed in a Philips 301 electron microscope.

Results

Water-soluble proteins

Freeze-fracture images of very small proteins, such as cytochrome *c* and ribonuclease, showed only large smooth fracture surfaces devoid of any morphological features, identical to those of the corresponding buffer solutions without proteins (Fig. 1). For larger proteins, smooth fracture surfaces were covered with randomly dispersed particles (Fig. 1). These particles were discernible in the case of myoglobin, and they were very clearly visible for hemoglobin and larger proteins. The distribution of particle sizes, as measured in a direction perpendicular to their shadows, was characteristic for each protein and, except for myoglobin, exhibited a close to Gaussian distribution, as demonstrated by the histograms for hemoglobin and catalase shown in Fig. 2. In the case of myoglobin small sizes (less than 40 Å) are cut off from the histogram, due to the limited resolution of the method (see Fig. 2). The distributions are consistent with the absence of any marked asymmetry in the structure of the examined proteins, but further analysis of the histograms in terms of the shape of the proteins is not possible. This would require knowledge of the mechanism of fracture of proteins in solution which is not available at present (see Discussion). However, we have compared the relation between the Stokes diameter and the median value of particle sizes obtained by freeze fracture (Fig. 3). It is seen that the mean particle size observed by freeze fracture increases with increasing dimensions of the protein. However, the slope of the regression line is less than unity and intersects the ordinate above zero. This means that this representation of freeze-fracture data overestimates the size of small proteins, while the mean particle size is lower than the Stokes diameter for large proteins (see Discussion).

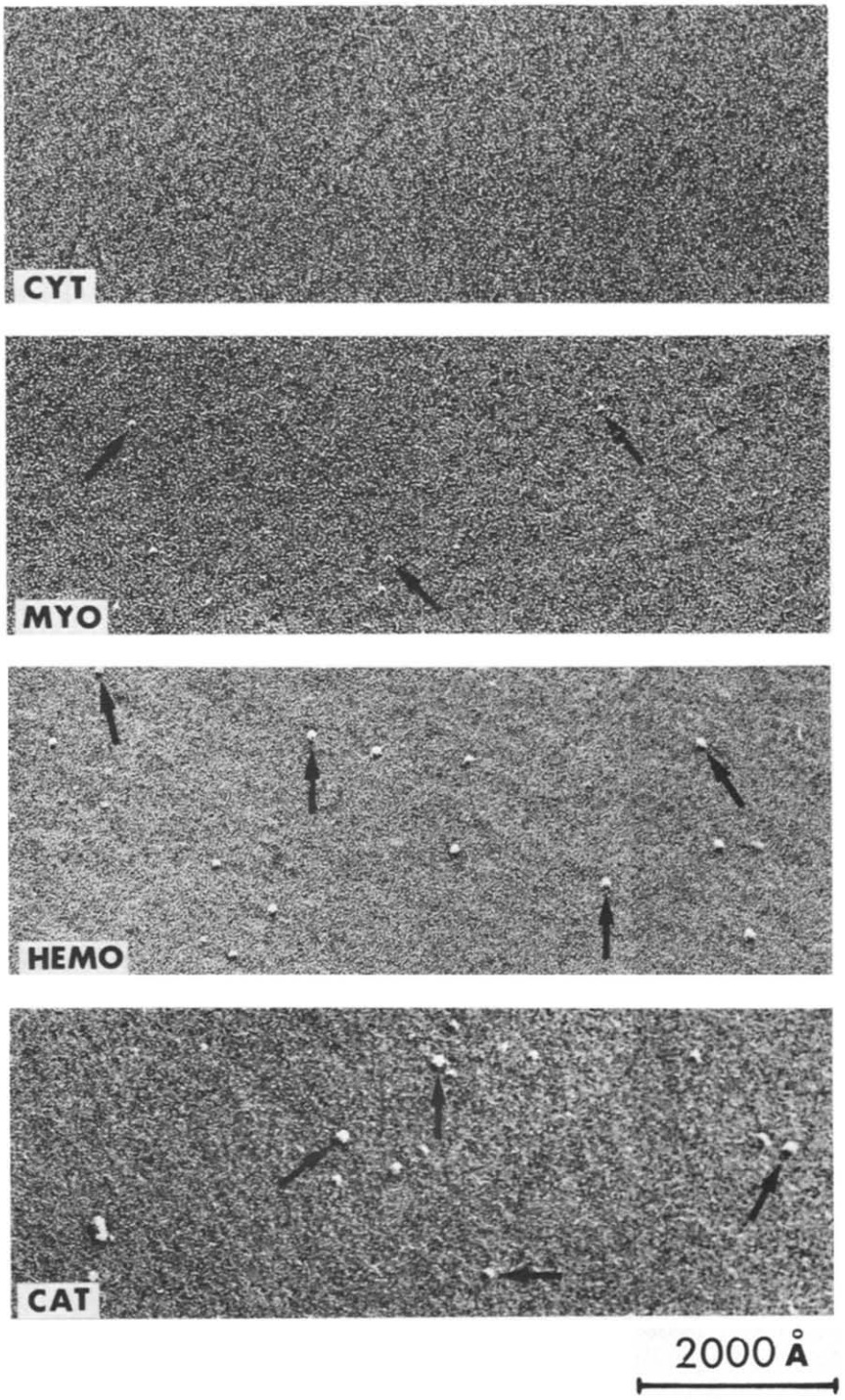


Fig. 1. Freeze-fracture images of four standard water-soluble proteins. The concentration of all proteins was about 10 mg/ml. Cytochrome *c* (M_r 13 400) is too small to be revealed by freeze-fracture. Myoglobin (M_r 16 900) is revealed as small, randomly dispersed particles which are quite difficult to see against the background. Hemoglobin (M_r 64 000) and catalase (M_r 220 000) are very clearly visible as randomly dispersed particles. All pictures are at magnification of 135 000×.

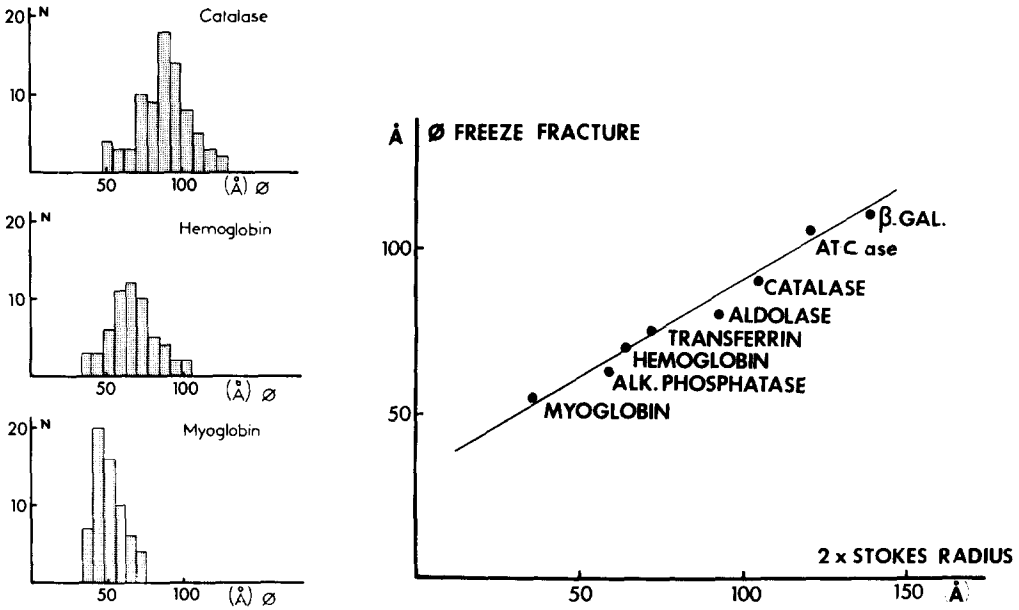


Fig. 2. Histograms representing the size distribution of particles corresponding to myoglobin, hemoglobin and catalase. N represents the number of particles counted and ϕ is the width of particles as measured in a direction perpendicular to their shadows. The measurements were done on micrographs similar to those shown in Fig. 1.

Fig. 3. Correlation between the mean values of the sizes of particles, as measured from histograms similar to those shown in Fig. 2, and the Stokes diameter, as determined from combined sedimentation equilibrium and sedimentation velocity data (Table I of Ref. 3). ATCase, aspartate transcarbamylase.

Sarcoplasmic reticulum Ca^{2+}-ATPase

In Fig. 4 are shown freeze-fracture images of different fractions of deoxycholate-solubilized ATPase isolated by gel chromatography (Fig. 5) together with images of sarcoplasmic reticulum before and after solubilization with detergent. The figures show that each of the seven preparations studied has its own characteristic morphology: (1) Sarcoplasmic reticulum preparations show vesicles displaying smooth convex and particulated concave fracture faces, due to the asymmetric insertion of the Ca^{2+}-ATPase in the membrane [11—20]. (2) Solubilized sarcoplasmic reticulum before column chromatography shows the presence of randomly distributed particles of different sizes, but mainly monomers, in agreement with a previous analytical ultracentrifugation study [7]. (3) The void volume fraction shows highly aggregated particles. (4) Trimer, dimer and monomer fractions show randomly distributed particles, some of which are aggregated into trimers in trimer fractions, into dimers in dimer fractions and without visible aggregation in the monomer fraction. (5) Phospholipid-detergent mixed micelles and detergent micelles show only smooth fracture faces, devoid of any morphological features. The second qualitative conclusion which can be drawn from the observation of the images is the high morphological homogeneity of the monomer fraction and the more complex nature of dimer and trimer fractions. The quantitative analysis of these three fractions is shown

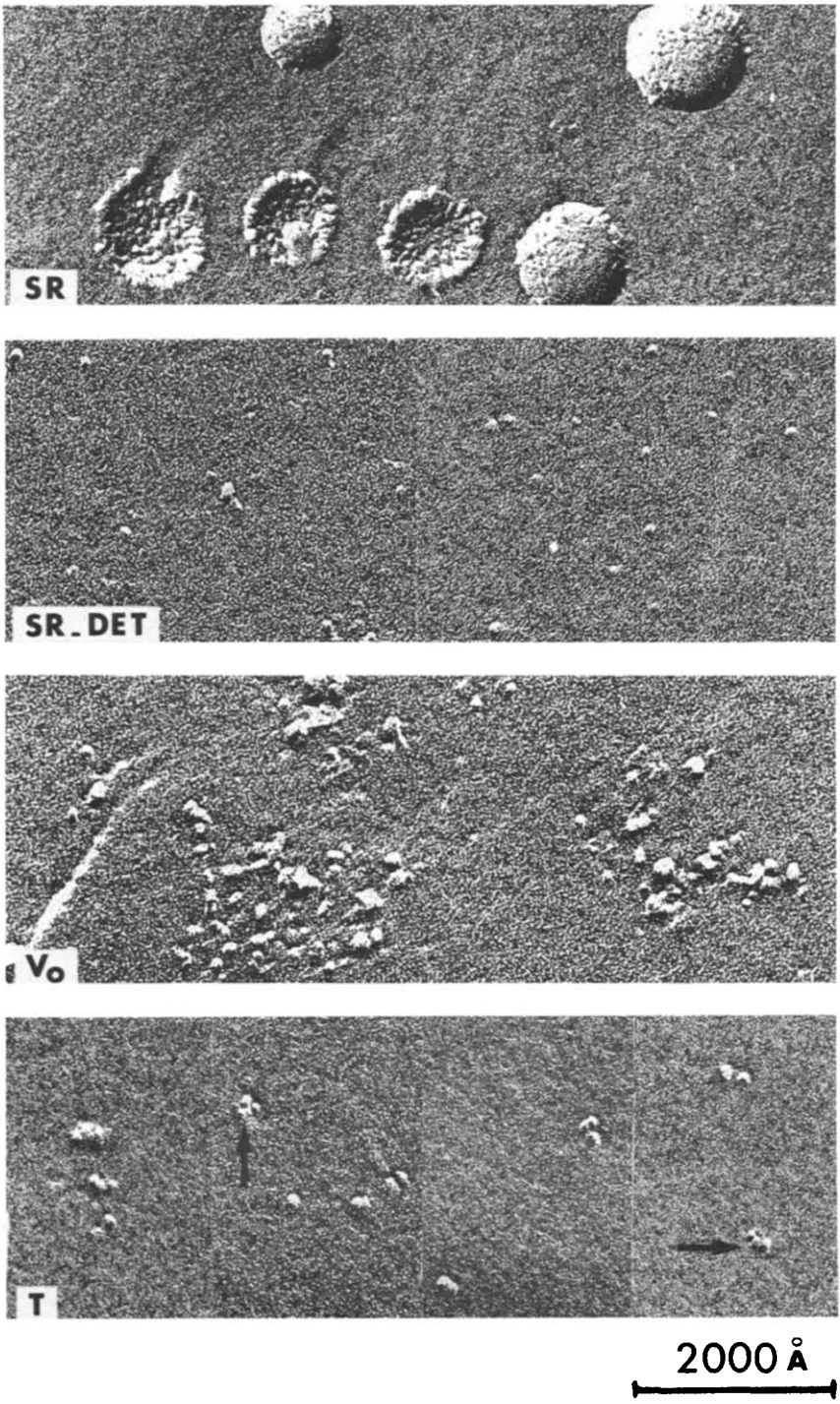
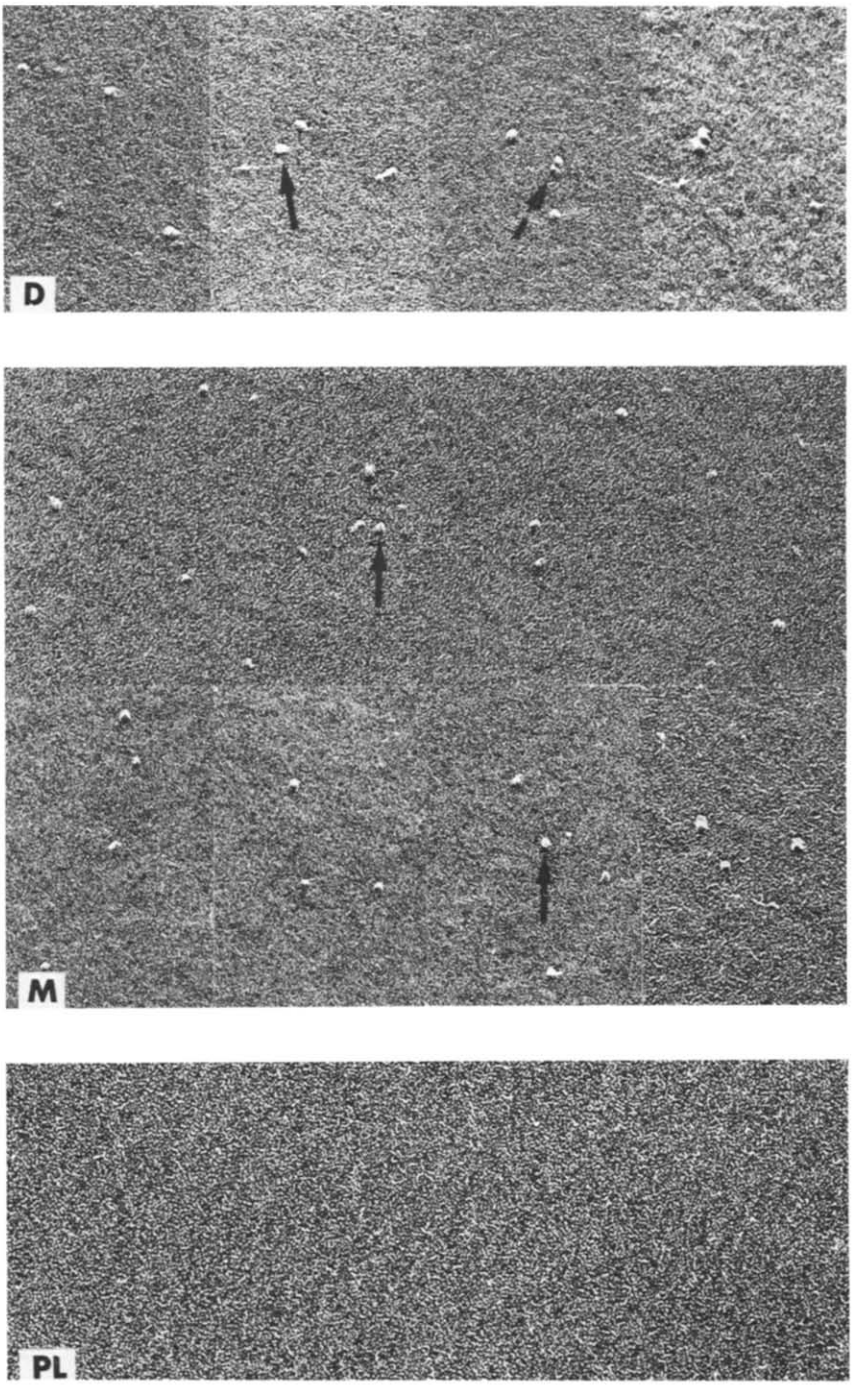


Fig. 4. Freeze-fracture images obtained from samples during the various steps of solubilization and purification of Ca^{2+}-ATPase into different soluble forms by gel chromatography (see Fig. 5). The abbreviations are the following: SR, native sarcoplasmic reticulum; SR-DET, sarcoplasmic reticulum solubilized by deoxycholate and prior to gel chromatography; V_0, void volume of the column eluant (aggregated proteins); T, trimer or tetramer fraction; D, dimer fraction; M, monomer fraction; PL, mixed phospholipid-



detergent micelles. Note the different morphological appearance of each fraction. The pictures of soluble forms of Ca^{2+}-ATPase are composite because of the low concentration of proteins in the fractions used for the freeze-fracture studies. All pictures are shown at a magnification of 135 000×.

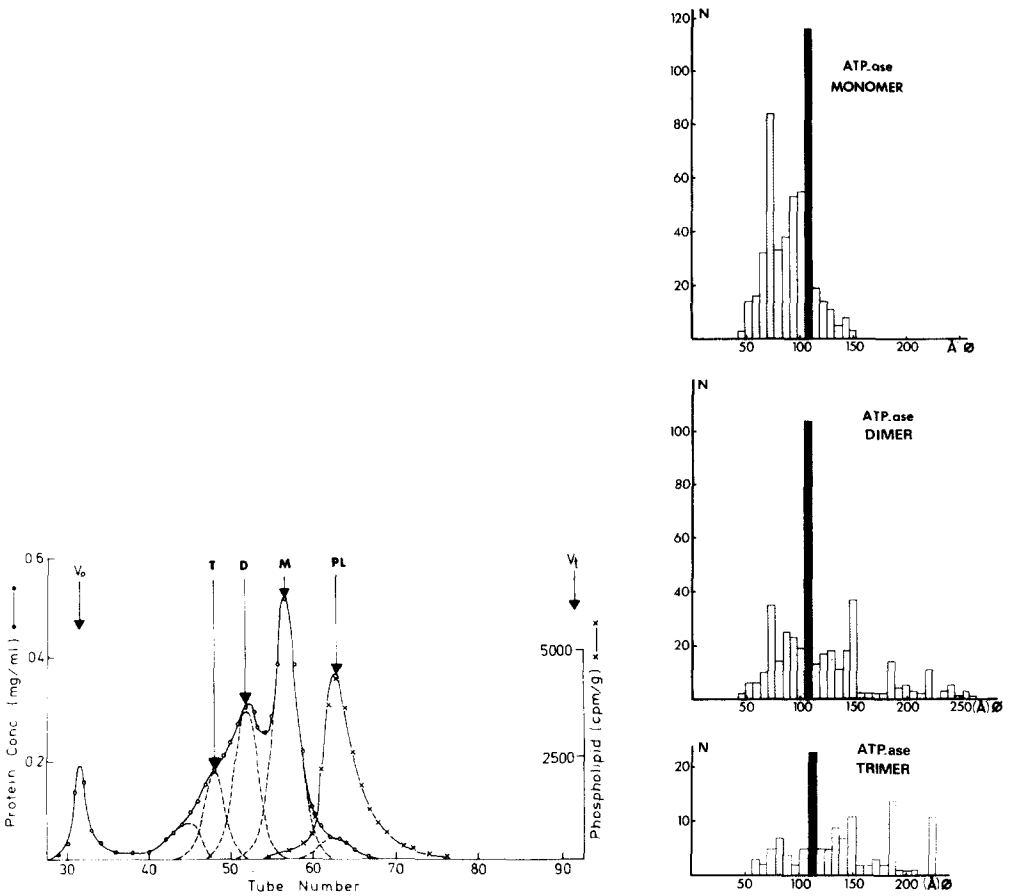


Fig. 5. Example of preparation of soluble forms of Ca^{2+}-ATPase by gel chromatography [7]. Vesicles of ATPase (1 ml of 8 mg protein/ml), suspended in 1 mM Hepes (pH 7.5)/0.015 M dithiothreitol/0.3 M sucrose were solubilized by addition of 0.205 M deoxycholate to a detergent/protein ration of 1.5/1. Solubilization at 20°C took place in a test tube containing a trace of [U-^{14}C]phosphatidylcholine. 5 min later the resultant mixture was applied to a Sepharose 6B column (1.5 × 90 cm) and eluted in the presence of 5 mM deoxycholate. The 5 mM deoxycholate eluant medium in addition to the detergent contained 0.03 M Tris-HCl (pH 8.3), 1 mM EDTA and 1 mM azide. The arrows designate the following: V_0, void volume of the column; T, trimer or tetramer fraction; D, dimer fraction; M, monomer fraction; PL, mixed micelles of deoxycholate and phospholipid; V_t, total volume. The broken lines represent an attempted resolution of the elution profiles of soluble ATPase into constituent components. The physicochemical characterization of these components is described in Ref. 7. Fractions corresponding to the different fractions were pooled, concentrated and analyzed by freeze-fracture electron microscopy (Fig. 4).

Fig. 6. Histograms representing the size distribution of particles of different soluble forms of Ca^{2+}-ATPase. N is the number of counted particles, and ϕ is the dimension of particles as measured in a direction perpendicular to their shadows. The measurement was done on micrographs similar to those shown in Fig. 5. Note the existence of distinct peaks in all three histograms. The highest peak in all three fractions is found at the same place (at about 110 Å). The first peak of monomer (at 75 Å) is also present in the preparations of dimer and trimer in addition to the peak corresponding to its double value in dimer (at 150 Å) and its triple value in trimer (at about 230 Å). This distribution of particle sizes is discussed in the text in terms of Ca^{2+}-ATPase dimensions (see Discussion).

in Fig. 6, where the distribution of the particle sizes, as measured in a direction perpendicular to their shadows, is presented. The most interesting feature of these histograms is the presence of two distinct peaks in the monomer fraction (at about 75 Å and 110 Å) which are also present, although with different ratios of heights, in dimer and trimer fractions. In the dimer fraction a third peak is present at about 150 Å, i.e. a length twice that of the first monomer peak. In the trimer fraction two additional peaks are present, as compared to the dimer, situated at about 180 Å and 230 Å (about three times the dimension of the first monomer peak). These types of histogram are clearly different from those observed for globular proteins, and they suggest that the shape of Ca^{2+}-ATPase monomers and oligomers is more complex (see Discussion).

We have also examined other types of preparations, giving well-defined monomers or oligomers of ATPase. By solubilization of ATPase in presence of 0.3 M sucrose and 0.4 M KCl [8] we obtained an enzymatically active preparation with the same appearance as the gel-chromatographed monomer. Similarly, by the use of $C_{12}E_8$ as a solubilizing detergent [6,9] we prepared monomers and trimers which had the same appearance as the corresponding deoxycholate-solubilized ATPase molecules.

Discussion

The data presented in this communication indicate that conventional freeze-fracture electron microscopy can be used for morphological characterization of macromolecules in solution. The presence of such molecules is indicated by the appearance of particles (when the molecules are at least as large as myoglobin) that are randomly dispersed, in the case of homogenous preparations, or which display a more complex distribution in the presence of aggregates. The quantitative data on the distribution of particle sizes may provide some information about the approximate dimensions of the molecules. The broadness of this distribution is partly related to the variation of particle sizes, due to fractures at various depths of molecules and/or of different widths of the randomly oriented molecules with respect to the shadowing beam. In addition, particle sizes are dependent on the grain size of the shadowing material and on possible decoration and plastic deformation artefacts [21]. The latter type of contribution concerns the performance of the freeze-fracture technique itself, and its effect can be minimized by using improved procedures of fracturing and replication, such as, e.g. very low temperature fracturing at high vacuum, followed by W-Ta shadowing [21]. A direct interpretation of histograms in terms of dimensions of molecules, furthermore, requires a detailed knowledge of the fracture mechanism of molecules in solution. Even in the absence of such a knowledge, one can still obtain valuable information about the approximate dimensions of a given molecule by comparison of its freeze-fracture histogram with those of molecules of known sizes, or by the use of a calibration curve, such as the one shown in Fig. 3. It is clear from this figure that, for globular proteins, there is a fair agreement between the measured mean particle size and Stokes diameter of corresponding proteins in the range of 60—110 Å. The overestimation of the sizes of smaller proteins can be ascribed, in part, to the low resolution of the method (which does not allow visualization of particle sizes smaller than 40 Å)

and to a quite large grain of the shadowing material (more than 20 Å for Pt-C shadowing).

The apparent underestimation of the sizes of large molecules is probably related to the choice of mean pearticle size as a parameter representing the diameter of particle. This parameter was mainly chosen because it was the easiest one to measure with good precision, but it is evident that, especially for large molecules, the mean freeze-fracture width is expected to be smaller than the true value of the molecular diameter because of the contribution to histograms of particles reflecting molecules fractured at various depths.

The results obtained on detergent-solubilized ATPase revealed several interesting features. First of all, in contrast to the globular proteins, the distribution of particle sizes of the monomer was bimodal with peaks occurring at 75 and 110 Å (Fig. 4). This preparation previously was found to give a single, homogenous boundary by analytical ultracentrifugation [7], so it is improbable that we are observing two types of monomeric ATPase. It is more likely that the bimodal distribution originates from the fracture of molecules with a characteristic shape, different from that of globular proteins. The width corresponding to the second peak agrees well with the maximal length of the deoxycholate monomer which we have determined to be 115 Å by low angle X-ray studies (Le Maire, M., Møller, J.V. and Tardieu, A., unpublished results). A value of 110 Å for the length of the particle also agrees with measurements by negative staining of solubilized ATPase [22] and of the total thickness of the sarcoplasmic reticulum membrane after tannic acid/osmium tetroxide staining [23].

The existence of a peak at 75 Å could indicate that this is a characteristic dimension of ATPase in a direction which is different from the long axis of the molecule. The most obvious possibility would be a cylindrical object with a diameter of 75 Å and a length of 110 Å. However, the volume of such an object is about twice that of the deoxycholate-solubilized monomer, including bound deoxycholate and solvent (unpublished results). On the basis of the scattering properties of ATPase, and taking into account information indicating an asymmetric distribution of ATPase in the membrane, we have proposed another model for the shape of the monomer (Le Maire, M., Møller, J.V. and Tardieu, A., unpublished results). According to this model the hydrophilic portion is rather bulky and has a width of around 75 Å, while the hydrophobic portion is long and thin. For such a shape the more bulky, hydrophilic head might be expected to dominate the distribution pattern in fractures perpendicular to the long axis of the molecule, giving rise to a peak in the histogram at around 75 Å.

The histograms of dimers and trimers showed that some of the particles had the same dimension as the monomer, perhaps because only one polypeptide in the oligomer was exposed after fracture. Particle widths exceeding the dimensions of the monomer were also characterized by the presence of peaks in the histograms. The presence of one additional peak in solution of dimers at the dimension of about twice that of the first peak of monomer, and in solution of trimers at the dimension of about three times of the same peak, can be interpreted as reflecting the presence of linear association of monomers into dimers and trimers, respectively. The presence of a distinct peak at 180 Å in the solution of trimers can perhaps be accounted for by non-linear association of

monomers (head-to-tail). In agreement with the idea of different modes of aggregation of oligomers, sedimentations in the analytical ultracentrifuge of such preparations give rise to heterogenous boundaries [9].

A firm interpretation of the freeze-fracture particles in terms of molecular shape is hampered by the fact that different models can be proposed to account for a given distribution of particle sizes, and by the lack of knowledge concerning the mechanism of fracture of proteins in solution. In the case of Ca^{2+}-ATPase an additional difficulty comes from the presence of detergent bound to the protein, although it seems probable that the detergent is removed from the protein during fracturing. In any event the amount of bound deoxycholate is low (approx. 0.2—0.3 g/g protein [7]), so that the possible presence of detergent on the fractured particles is not expected to have any major effect on the shape properties of the ATPase.

As far as the shape of molecules in solution is concerned, its direct visualization by freeze-etching technique will require the use of ultrarapid freezing of solutions without glycerol, followed by controlled etching and highly improved shadowing conditions. In spite of great effort and many technological improvements it is still difficult to obtain, in a reproducible way, such images.

Acknowledgments

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